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QUANTITATIVE MICROANALYSIS OF CAPSAICIN, DIHYDROCAPSAICIN AND NORDIHYDROCAPSAICIN USING MASS FRAGMENTOGRAPHY

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SUMMARY

A mass fragmentographic method for the quantitative microanalysis of capsaicin, dihydrocapsaicin and nordihydrocapsaicin in the fruits of *Capsicum annuum* has been developed. The molecular ions at *m/e* 377, 379 and 365 in the mass spectra were used for monitoring the trimethylsilyl derivatives of capsaicin, dihydrocapsaicin and nordihydrocapsaicin, respectively. The ratios of the height of each molecular ion to that of an internal standard (cholestane) were linear over the range 5–60 ng. The purification of individual capsaicinoids by high-performance liquid, thin-layer and gas–liquid chromatography is also described.

INTRODUCTION

Capsaicin is a pungent principle of red peppers, the fruits of *Capsicum annuum* and *Capsicum frutescens*. Many workers have studied capsaicin because of its importance in spices, food additives and drugs. The structure of capsaicin has been established as N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-*trans*-6-enamide[1,2]. Recently, it has been shown by mass spectrometry (MS)[3–5] and gas chromatography–MS[6] that the natural material is a complex mixture of the closely related amides; capsaicin, dihydrocapsaicin (DC), nordihydrocapsaicin (NDC), homocapsaicin (HC) and homodihydrocapsaicin (HDC), the structures of which are the vanillylamide of 8-methylnon-*trans*-6-enoic acid, 8-methylnonanoic acid, 7-methyloctanoic acid, 9-methyldec-*trans*-7-enoic acid and 9-methyldecanoic acid, respectively. In this paper, the term "capsaicinoid(s)" will be used to represent these homologues and analogues of capsaicin.

In the course of our studies on the biosynthetic pathway of capsaicinoids in *Capsicum* fruits, it became necessary to determine the individual compounds separately. The quantitative determination of total capsaicinoids has been achieved by gas–liquid chromatography (GLC)[7,8], thin-layer chromatography (TLC)[9,10] and ultraviolet[11] and colorimetric methods[12,13]. The sensitivities of these methods are at the

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microgram level. In order to determine the individual capsaicinoids, a GLC method has been developed in recent years[6,14,15]. In this method, trimethylsilyl (TMS) derivatives were prepared and analyzed. The separation of TMS-capsaicin and TMS-DC by GLC, however, has not been satisfactory and the sensitivity of GLC itself was not great enough for micro-determination at the nanogram level.

This paper describes a method for the quantitative microanalysis of the individual members of capsaicinoids in *Capsicum* fruits by mass fragmentography (MF), which allows the simultaneous and quantitative determination at 5–60-ng levels of individual members of the capsaicinoids in incompletely resolved GLC peaks. The purification of individual members by high-performance liquid chromatography (HPLC), TLC and GLC is also described.

EXPERIMENTAL

Reagents and materials

N,O-Bis(trimethylsilyl)acetamide in acetonitrile (TMS–BA, Cat. No. B-0510) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 5-α-Cholestane and other chemicals were purchased from Nakarai Chemicals (Kyoto, Japan).

Air-dried red fruits of *Capsicum annuum* native to Japan ("Takanotsume") were purchased from a local market in Kyoto, Japan. Air-dried red fruits of *C. annuum* native kinds to Korea ("I-Sung") were obtained from Taegu, Korea.

Capsaicin, DC and NDC were purified from "Capsaicin" (Sigma, St. Louis, Mo., U.S.A.; Cat. No. M9253) by using HPLC or TLC and GLC. The details are given below.

Nuclear magnetic resonance (NMR) measurements

The NMR spectra were obtained at room temperature with a Varian EM-360 NMR spectrometer with tetramethylsilane as internal reference. Samples (20 mg) were dissolved in deuterated chloroform. Capsaicinoids were analyzed according to the method of Müller-Stock *et al.*[16].

Separation of capsaicin and DC by HPLC

HPLC was performed at a pressure of 70 kg/cm^2 on a stainless-steel column (1 m × 2.1 mm I.D.) packed with Permaphase ODS (25-37 μm) (octadecyltrimethoxysilane; DuPont, Wilmington, Del., U.S.A.) by using a Shimadzu–DuPont 830 high-pressure liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan). Elution was carried out at room temperature with a linear gradient using 30% methanol in water in the mixing chamber by adding 99.5% methanol at a rate of 2% per minute.

Sample solution (1 μl) containing 60 μg of the capsaicin mixture dissolved in chloroform was applied on to the column and monitored at 254 nm.

Separation of capsaicin and DC by TLC

TLC was carried out according to the method of Kosuge and Furuta[4]. Capsaicin mixture (200 μg) was loaded on a 20 × 20 cm silica gel G plate, 0.25 mm thick (Merck, Darmstadt, G.F.R.), impregnated with 10% tetralin in diethyl ether and developed with methanol–2% silver nitrate solution (1:1, v/v). Capsaicinoids in two peripheral pilot spots were located by spraying with 0.1% 2,6-dichloroquinone-4-

chloroimide solution[5]. The zones corresponding to the individual members were scraped off and extracted into the chloroform–methanol solution[17]. After washing with distilled water and dehydration, the chloroform solution was evaporated to dryness *in vacuo*.

GLC of TMS derivatives of capsaicinoids

The capsaicinoids obtained by HPLC or TLC were trimethylsilylated by reaction with an excess of TMS–BA at room temperature for 3 h. The reaction mixture was evaporated to dryness under nitrogen gas and the residue was dissolved in a known amount of ethyl acetate dried with sodium sulphate. The GLC of TMS derivatives of capsaicinoids was carried out on a Shimadzu Model GC-5A gas chromatograph, equipped with a flame-ionization detector (Shimadzu Seisakusho). The separation column used was a glass column (1.5 m × 3 mm I.D.) packed with 3% silicone SE-52 on Chromosorb W (60–80 mesh, acid washed, silanized; Nishio Kogyo, Tokyo, Japan). The column was conditioned at 280 °C for 48 h before the analysis. The temperatures of the column, the injection port and the detector were 230°, 260° and 260°, respectively. The nitrogen flow-rate was 40 ml/min and the column inlet pressure was kept at 6 kg/cm^2.

GC–MS of TMS derivatives of capsaicinoids

The GC–MS of TMS derivatives of capsaicinoids was carried out on a Shimadzu–LKB Model 9000 gas chromatograph–mass spectrometer (Shimadzu Seisakusho). A total ion collector was used as a detector for GC–MS. The separation column used was a coiled glass column (2 m × 3 mm I.D.) packed with 3% silicone SE-52 on Chromosorb W. The temperatures of the column, the molecular separator and the ion source were 250°, 270° and 290°, respectively. The ionizing voltage was 70 eV, the accelerating voltage 3.5 kV and the trap current 60 μA. The gain of the mass spectrometer was 2, the slits were adjusted to 0.1 mm, the scan speed was 7, the oscillograph chart was run at 10 cm/sec and the filter was selected at 120 Hz. The helium flow-rate was 30 ml/min and the column inlet pressure was 1 kg/cm^2. The percentage total ion intensities of molecular ions at a low ionizing voltage were determined at 20 eV and calculated with a Shimadzu GC–MS PAC 300-D on-line computer system.

MF of TMS derivatives of capsaicinoids

MF was carried out on a Shimadzu–LKB 9000 gas chromatograph–mass spectrometer, equipped with a 9060S high-speed multiple ion detector–peak matcher (MID–PM) (Shimadzu Seisakusho). The mass spectrometer was adjusted to record the intensity at *m/e* 365 for TMS-NDC, *m/e* 377 for TMS-capsaicin, *m/e* 379 for TMS-DC and *m/e* 372 for 5-α-cholestane as internal standard. The MF conditions were as follows: gains, 2 for GC–MS and 5.0 for MID–PM; filter, 20 Hz; ionizing voltage, 20 eV; oscillograph chart speed, 10 mm/min. Other GC–MS conditions were as described in the GC–MS section.

Procedure

A 1–5-g amount of the powder of dried fruits of *Capsicum annuum* was extracted three times with 100-ml portions of acetone in a Waring blender, followed by

extraction three times with 100-ml portions of diethyl ether, until the residue became colourless and no capsaicin was detected by a colour reaction[12]. The extracted solution was evaporated *in vacuo* and the oily material obtained was re-extracted three or four times with 1-ml portions of acetone. The extract was evaporated *in vacuo*, and made up to 1–5 ml with acetone. After a preliminary determination of amounts of capsaicinoids by TLC[9], 0.1–3 μg of capsaicinoids in the extract were purified by TLC on a silica gel G plate by development with chloroform–ethanol (98:2)[9]. Capsaicinoids in two peripheral pilot spots were located by using 0.1% 2,6-dichloroquinone-4-chloroimide solution[5]. The zone corresponding to the capsaicin mixture was scraped off the plate. To the powder, 100–250 μl of the internal standard solution, in which 5-α-cholestane was dissolved in ethyl acetate at a concentration of 2 μg/ml, was added. The mixture was extracted five times with 2-ml portions of ethyl acetate and the extracts were collected in a 5-ml screw-capped vial (Wheaton Scientific) and evaporated to dryness *in vacuo*. Trimethylsilylation was carried out in the vial as described above. The solution (1–3 μl) was injected into the gas chromatograph–mass spectrometer for MF.

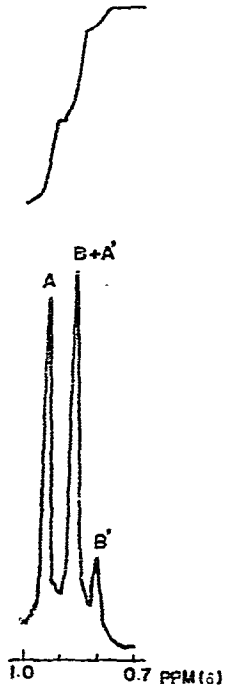

Fig. 1. NMR spectrum of Sigma "Capsaicin". Peaks A and B are the doublet corresponding to the olefinic components and A' and B' are the doublet corresponding to the saturated components.

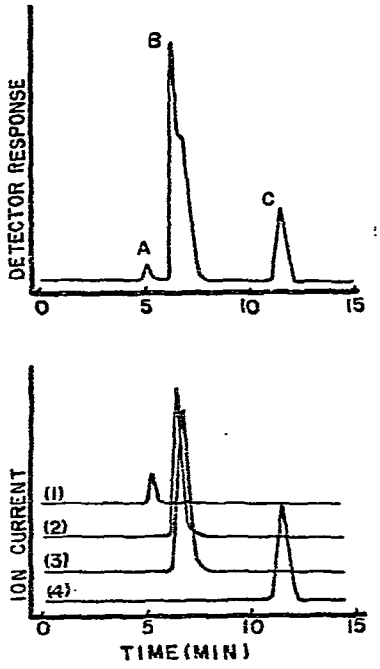

Fig. 2. Gas chromatogram (above) and mass fragmentogram (below) of capsaicinoids of Sigma "Capsaicin". Peaks A, B and C correspond to TMS-nordihydrocapsaicin, mixture of TMS-capsaicin and TMS-dihydrocapsaicin, and 5-α-cholestane (internal standard), respectively. The mass spectrometer was set to detect the molecular ions of TMS-nordihydrocapsaicin [(1), *m/e* 365], TMS-capsaicin [(2), *m/e* 377], TMS-dihydrocapsaicin [(3), *m/e* 379], and 5-α-cholestane [(4), *m/e* 372].

To determine the recovery of the extraction procedure, 1 mg of authentic capsaicin was added to 1 g of ground green fruits of *C. annuum*, var. "Shishito", in which no capsaicin was detected. After the extraction, TLC separation and trimethylsilylation, the sample was analyzed by GLC. The recovery of added capsaicin was 93.6%. When authentic capsaicin was added prior to the TLC separation, the recovery was 98%.

RESULTS

The Sigma "Capsaicin" was found to be a mixture of olefinic components (74.6%) and saturated components (25.4%) by NMR measurement according to the method of Müller-Stock *et al.*[16], as shown in Fig. 1. GLC analysis of the TMS derivative of Sigma "Capsaicin" showed the presence of three components, *viz.*, TMS-capsaicin, TMS-DC and TMS-NDC. However, the separation of TMS-capsaicin and TMS-DC by GLC was not sufficiently good to allow the quantitative determination of each component (Fig. 2). MF gave the completely separated peaks (Fig. 2). In MF, however, a calibration factor, the ratio of the percentage total ion intensity of each molecular ion, was required in order to determine the amount of each component.

In order to obtain this ratio, each component was first purified, by using HPLC and GLC, or TLC and GLC. HPLC was carried out on the Permaphase ODS column to separate capsaicin and DC. Fig. 3 shows the chromatograms obtained by HPLC of Sigma "Capsaicin", on which two peak fractions are evident. Each fraction was collected, evaporated *in vacuo*, and identified by GLC–MS after the TMS–BA treatment. Fraction I was identified as a mixture of capsaicin and DC, and fraction II as DC. Capsaicin and DC in fraction I were separated completely by GLC. Fig. 4 shows the gas chromatograms of fractions I and II obtained by HPLC. Fig. 5 shows the mass spectra of the TMS derivatives of capsaicin, DC and NDC separated by HPLC and GLC. The results demonstrate that the three components obtained by the above procedures are mass spectrometrically pure.

By use of the pure capsaicin, DC and NDC, total ion intensities of the molecular ions of these capsaicinoids were measured. The intensities of the molecular ions of capsaicinoids at 20 eV were greater than at 70 eV. The mean values obtained in triplicate experiments are shown in Table I. The total ion intensities of the molecular ions of TMS-capsaicin, TMS-DC and TMS-NDC were 14.2, 25.9 and 26.7%, respectively. In order to estimate the ionization ratios of capsaicin, DC and NDC, the purified components were analyzed by GLC and MF. It was found that the ionization ratio, the ratio of the total ion intensities for equal amounts of TMS-capsaicin, TMS-DC and TMS-NDC, was 1:1:1.

The standard graphs for the quantitative determination of the capsaicinoid were prepared by using 5-α-cholestane as an internal standard. A standard solution of 5–60 ng/μl of capsaicinoids containing 10 ng of cholestane was prepared and trimethylsilylated and analyzed by MF as described under Experimental. The elution position of cholestane is shown in Fig. 2. As shown in Fig. 6, the ratios of the height of the molecular ion to that of the internal standard were linear over the range 5–60 ng. If the gain control is adjusted, about 1 μg of capsaicinoids could also be analyzed.

Using the technique described above, Sigma "Capsaicin" was analyzed and, from the results of MF analysis, it was found to consist of 71% capsaicin, 26% DC,

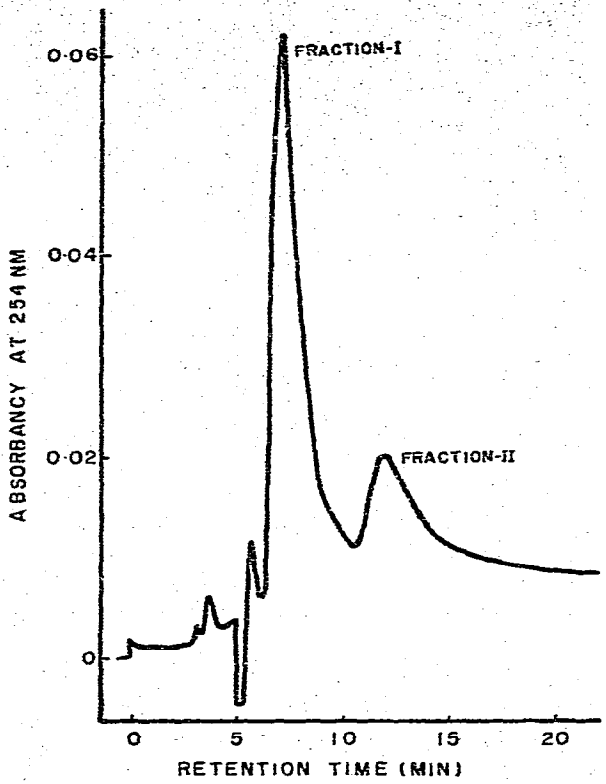

Fig. 3. Chromatogram of Sigma "Capsaicin" obtained by high-performance liquid chromatography.

and 3% NDC. The ratio of the olefinic components to the saturated components was 71:29. This result agrees closely with the data obtained by NMR spectrometry.

The composition of the capsaicinoids in the extracts from *Capsicum* "Takanotsume" and "I-sung" fruits was also analyzed by the procedure presented above.

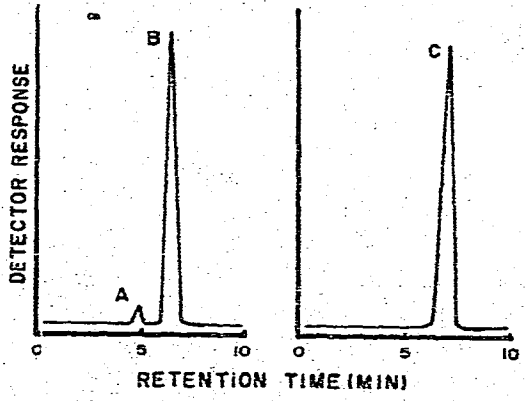

Fig. 4. Gas chromatograms of fractions I (left) and II (right) obtained by high-performance liquid chromatography. A, B and C were identified by mass spectrometry as TMS-nordihydrocapsaicin, TMS-capsaicin and TMS-dihydrocapsaicin, respectively.

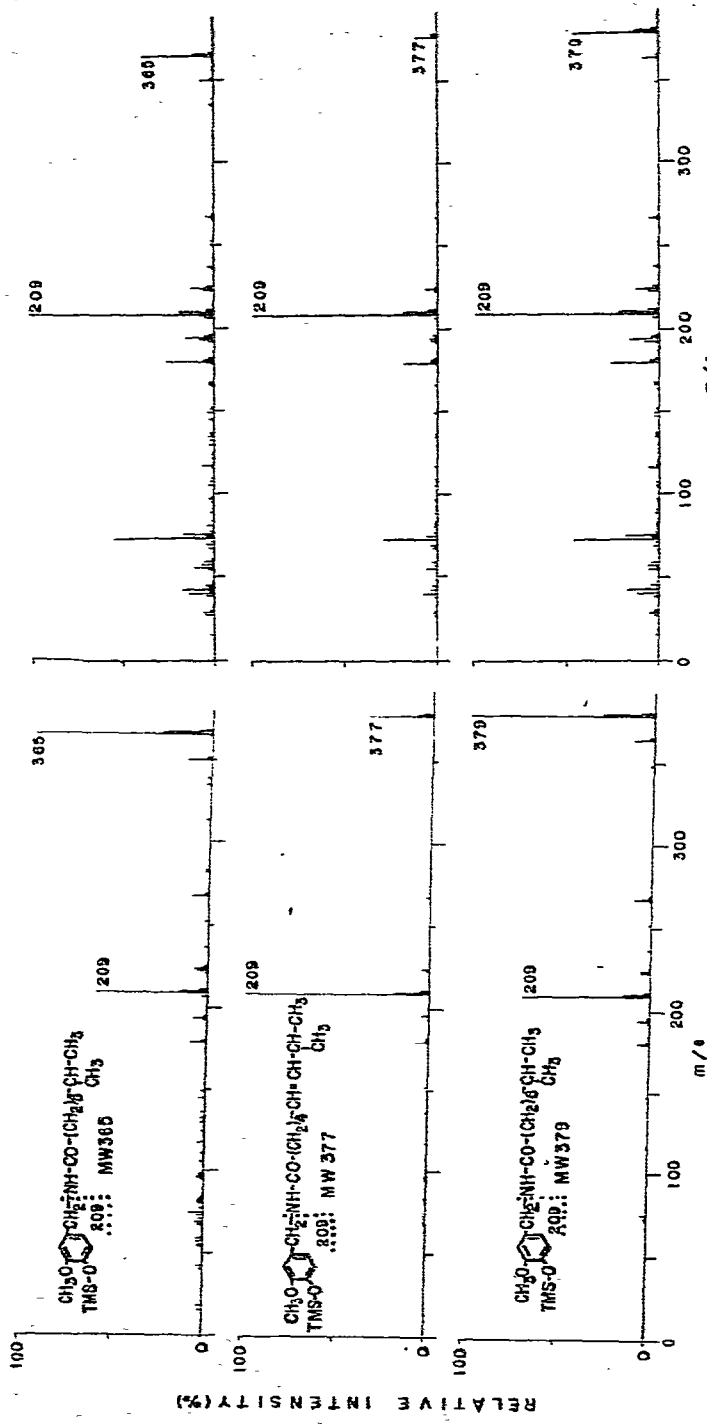

Fig. 5. Mass spectra of TMS-nordihydrocapsaicin (above), TMS-capsaicin (centre) and TMS-dihydrocapsaicin (below) separated by HPLC and GLC. The electron energy used was 20 eV (left) and 70 eV (right).

TABLE I

RELATIVE AND TOTAL ION INTENSITIES OF PRINCIPAL IONS OF TMS DERIVATIVES OF CAPSAICINOIDS

TMS derivative	*m/e*	*Relative intensity (%)*	*Total ion intensity (%)*
TMS-capsaicin			
M^+	377	32.9	14.2
$[M-15]^+$	362	0	0
$[M-RNH]^+$	209	100	41.5
TMS-DC			
M^+	379	100	25.9
$[M-15]^+$	364	12.2	3.1
$[M-RNH]^+$	209	79.2	19.3
TMS-NDC			
M^+	365	100	26.2
$[M-15]^+$	350	13.0	3.5
$[M-RNH]^+$	209	70.9	16.7

TABLE II

CONTENTS OF CAPSAICINOIDS IN THE CAPSICUM FRUITS OF "TAKANOTSUME" AND "I-SUNG"

Variety	*Capsaicin*		*DC*		*NDC*		*Total*	
	*μg/mg**	*%***	*μg/mg**	*%***	*μg/mg**	*%***	*μg/mg**	*%***
"Takanotsume"	1.89	45	1.68	40	0.63	15	4.2	100
"I-Sung"	0.92	38	1.15	48	0.34	14	2.4	100

* μg/mg of dry weight.

** Capsaicinoid as a percentage of the total amount.

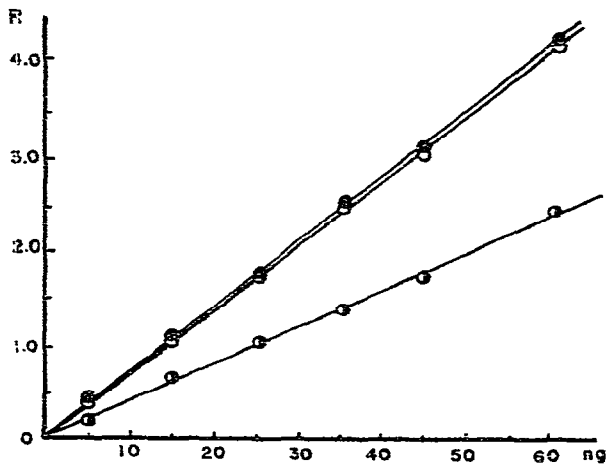

Fig. 6. Standard graphs for the quantitative determination of capsaicinoids by MF. R = ratio of peak heights of molecular ions of capsaicinoids to that of an internal standard. ●, TMS-nordihydrocapsaicin; ○, TMS-dihydrocapsaicin; ◐, TMS-capsaicin.

As shown in Table II, the proportions of capsaicin and DC in the total capsaicinoids in the fruits of "Takanotsume" (45 and 40%) were considerably different from those in "I-Sung" (38 and 48%, respectively). The percentage of NDC in "Takanotsume" was similar to that in "I-Sung". The total content of the capsaicinoids in "Takanotsume" was approximately twice as high as that in "I-Sung".

DISCUSSION

GLC has been used for the separation of capsaicinoids by many workers[6,14,15], but capsaicin and DC have not previously been separated successfully although various column conditions were applied.

On the other hand, MF has recently been used for the quantitative determination of various components and for the investigation of the metabolic process, because of its advantages of high sensitivity at nanogram or picogram levels and its high separation power towards peaks that are unresolved in GLC[18,19]. In order to determine the individual components of capsaicinoids quantitatively, it is necessary to measure the total ion intensity of the molecular ion of each component. The total ion intensities of the molecular ions of the TMS derivatives of capsaicin, DC and NDC were compared with one another, and the correction factor was calculated. It is difficult to obtain commercially pure components of individual analogues and homologues of capsaicin. As commercial Sigma "Capsaicin" contained DC and NDC in addition to capsaicin, the separation of each component was carried out.

HPLC has been used for the separation of various naturally occurring components at a similar sensitivity level to TLC[20]. An additional advantage of HPLC is that the preparative-scale separation could be achieved by altering the column conditions. As shown in the present work, HPLC gives a good separation of capsaicin and DC in the capsaicin mixture. Capsaicin and NDC could not be separated by HPLC, but no further attempts were made in this direction as they can be separated completely by GLC.

TLC was also used to separate capsaicin and DC. A silica gel G plate impregnated with 10% tetralin was used and developed with methanol–2% silver nitrate solution (1:1)[4]. The pattern of the TLC separation was the same as that of HPLC. Capsaicin (R_F 0.64) and DC (R_F 0.59) were separated completely by TLC. Capsaicin and NDC could not be separated by these methods, as demonstrated by GLC–MS.

Bennett and Kirby[3], using TLC, NMR and MS, found that the composition of the phenolic components of *C. annuum* was 69% capsaicin, 22% DC, 7% NDC, 1% HC and 1% HDC. Leete and Louden[21] reported that the phenolic fraction of *C. frutescens* contained 47% capsaicin and 53% DC. Müller-Stock *et al.*[14] determined the composition of capsaicinoids in the few capsaicin mixtures available commercially using GLC: the capsaicinoids were found to consist of 46–77% capsaicin, 21–40% DC, 2–12% NDC and 0–2% vanillyl-N-nonylamide as adulterant.

This is the first work in which capsaicin, DC and NDC, the major constituents of *C. annuum* var. "Takanotsume" and "I-Sung", have been determined quantitatively at the nanogram level using MF. HDC and HC, which are minor constituents of *Capsicum* fruits, were also detected by MF and identified by GLC–MS. MF analysis showed that the ratio of the ion intensity of HDC to that of DC in saturated components was 1:31, and that of HC to capsaicin in olefinic components was 1:80. In this work, we did not attempt to isolate HDC and HC.

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